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Retroviral Transfer of Human Mutated Thymidylate Synthase Gene into Hematopoietic Stem Cells for Protection from High-Dose Fluoropyrimidine Toxicity

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In the field of breast cancer, the significant cytoreduction followed by consolidation chemotherapy has been accepted to reduce disease relapse in breast cancer. However, myelosuppression still impose restrictions on the optimization of this treatment modality. Insertion of drug resistance genes into hematopoietic progenitor cells offers an additional approach to allow further dose intensification and treatment post transplant. We have generated and characterized human thymidylate synthase (TS) mutants, one of which resulted in a 15-fold increase Ki for 5-fluoro-2-deoxyuridylate (FdUMP) compared to the wild type TS. This mutant TSG52S transfectant confers a 97-fold resistance to fluorodeoxyuridine (FdUrd) in TS negative mouse cell lines. 5-fluorouracil (5-FU), is a commonly used drug in breast cancer treatment with significant myelosuppressive side effects. We hypothesize that after administration of high dose chemotherapy, transplanted progenitor cells transduced with the fluoropyrimidine drug resistance gene, TSG52S, will allow for higher doses of 5-FU to be given during maintenance therapy without significant myelosuppression possibly leading to improved cure rates. Therefore, the overall objective is to develop retroviral vectors and efficient viral transduction methods for introducing TSG52S into mouse and human hematopoietic progenitor cells and then to evaluate the level of resistance to myelosuppression from 5-FU this method produces.

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Introduction

In the field of breast cancer, the significant cytoreduction achieved by high-dose chemotherapy with autologous progenitor cell support or recombinant hematopoietic growth factors remains an important strategy. The role of consolidation after high dose chemotherapy has been accepted to reduce disease relapse in breast cancer. However, toxicities of the chemotherapeutic agents, in particular myelosuppression, still impose restrictions on the optimization of this treatment modality. Insertion of drug resistance genes into hematopoietic progenitor cells offers an additional approach to allow further dose intensification and treatment post transplant. We have generated and characterized human thymidylate synthase (TS) mutants, one of which resulted in a 15-fold increase Ki for 5-fluoro-2-deoxyuridylate (FdUMP) compared to the wild type TS (Tong et al). This mutant TSG52S glycine at position 52 was replaced with serine) transfectant confers a 97-fold resistance to fluorodeoxyuridine (FdUrd) in TS negative mouse cell lines. 5-fluorouracil (5-FU), is a commonly used drug in breast cancer treatment with significant myelosuppressive side effects. We hypothesize that after administration of high dose chemotherapy, transplanted progenitor cells transduced with the fluoropyrimidine drug resistance gene, TSG52S, will allow for higher doses of 5-FU to be given during maintenance therapy without significant myelosuppression possibly leading to improved cure rates. Therefore, the overall objective is to develop retroviral vectors and efficient viral transduction methods for introducing TSG52S into mouse and human hematopoietic progenitor cells and then to evaluate the level of resistance to myelosuppression from 5-FU this method produces.

Body

Specific Aims

We plan to:

1) to construct retroviral vectors which contain the mutated TS (G52S) with and without mutated dihydrofolate reductase (DHFR) and mutated TS (G52S) with enhanced green fluorescent protein (EGFP). DHFR and EGFP are selectable markers which improve detection of transduction.
2) use these constructs to determine the efficiency of gene transfer and their ability to confer 5-FU resistance in vitro, using 3T3 cells and an HL60 cells as test systems. Mouse bone marrow cells will be used for in vitro CFU-GM assay.
3) test the hypothesis that gene transfer of the mutated TS (G52S) into hematopoietic cells protects them from high dose chemotherapy related myelosuppression in a mouse model.
4) test and optimize conditions for transduction of CD34 separated human hemtopoietic progenitor cells (peripheral blood stem cells) using these constructs.

Key Research Accomplishments

We have completed the aim 1) during this award period.
We initially constructed retroviral vectors SFG-TS52-IRES-EGFP and SFG-TS52-IRES-F/S, and SFG-TS52-IRES-Neo to test TS52 (mutated TS gene) resistance to fluoropyrimidine. These construct conferred 3-fold 5FU resistance at ED50 in transduced NIH3T3 cells compared to control. We have previously generated the double mutant DHFR (F22/S31;F/S) which was one of the most promising drug resistant genes (Ercikan-Abali et al). We designed a fusion gene of TS52 and F/S cDNA to generate a fusion protein which confers both fluoropyrimidine and folate analogue resistance

specifically methotrexate (MTX). DHFR mutant F/S may be a promising selection gene when transduced into human CD34+ cells and teated with MTX in long term culture (unpublished). In addition, another reason for generating this fusion protein was that a single bifunctional enzyme containing both DHFR and TS exists naturally in protozoans such as Leishmania and Cryptosporidium and we hypothesized that this fusion protein might be active in humans.

Construction of a fusion protein containing F/S and TS52 mutants.
We have previously constructed a fusion protein containing F/S-EGFP in a pkT7 plasmid. This plasmid contained restriction sites which enabled to cleaved out EGFP fragment. We generated a TS52 fragment containing these restriction sites by PCR and subcloned this fragment into the pkT7 plasmid to generate a pkT7-F/S-TS52. this plasmid was digested to remove the F/S-TS52 fragment to subclone into pET-17 x b plasmid vector. The wild type TS was cloned into pET-17 x b plasmid vector as a control. These plasmids were transformed into E Coli BL21 (DE3) and protein was induced. The fusion protein was functional and kinetic characteristics were similar to that of the individual mutant enzymes.

Construction of retroviral vectors containing F/S-TS52.
We have previously constructed a retroviral construct SFG-TS52S containing NcoI/XhoI sites which was digested and TS52S fragment was removed. Digesting pet-28-F/S-TS51 plasmid to obtain F/S-TS52 fragment which was subcloned into SFG backbone vector to yield **SFG-F/S-TS52**. We have previously constructed SFG-EGFP-IRES-F/S which was excised to obtain a EGFP-IRES fragment. This was cloned into SFG-F/S-TS52 to yield **SFG-EGFP-IRES-F/S-TS52**. We have cloned SFG-TS52-IRES-EGFP previously and IRES-EGFP was excised. This fragment was cloned into SFG-F/S-TS52 vector to yield **SFG-F/S-TS52-IRES-EGFP**. The vectors were sequenced, and the plasmid was purified for transfection into packaging cell lines, gp-AM12 and gp-E86. We have generated amphotropic producer cell line by co-transfection with pSV2 Neo plasmid with either SFG-EGFP-IRES-F/S-TS52, SFG-F/S-TS52-IRES-EGFP, or SFG-F/S-TS52 plasmid. We found that SFG-F/S-TS52-IRES-EGFP transfected AM12 producer cell lines did not show bright fluorescence compared to SFG-EGFP-IRES-F/S-TS52 by fluorescence microscopy or FACS analysis.

Transduction of the virus into NIH3T3 cells to test cytotoxicity and perform molecular analysis.
The supernatant harvested from AM12-SFG-F/S-TS52 and AM12-SFG-EGFP-IRES-F/S-TS52 was infected onto NIH3T3 cells by MOI of 10 with polybrene, and selected by 5μM FdUrd for 10 days or TMTX 20nM for 3 days to eliminate the non-transduced cells. Transduced cells and non-transduced cells were plated on 96 wells with various concentration of either 5-FU or MTX, or both to measure cytotoxicity by XTT assay (Rodgers et al). The result showed 6-fold increase (ED50) in 5-FU cytotoxicity in NIH3T3 cells transduced with SFG-F/S-TS52 compared to control cells. 5-FU cytotoxicity in NIH3T3 cells transduced with SFG-F/S-TS52 (fusion) vector was comparable to the one transduced with SFG-TS52-IRES-F/S vector (6-fold increase). MTX cytotoxicity was tested in same cells and showed 192-fold increase (ED50) in NIH3T3 cells transduced with the fusion vector compared to the control. MTX resistance was increased in fusion vector transduced NIH3T3 cells (192-fold) compared to SFG-TS52-IRES-F/S transduced cells (96-fold). These cells were infected at MOI of 10 and there is a possibility that the drug resistance difference might be secondary to the gene copy number. Further analysis using Southern blot will be required to conclude this

result.
The Western blot was performed to test the TS gene expression of the SFG-F/S-TS52 vector. NIH3T3 cells infected with SFG-F/S-TS52, control cells, and pure human TS protein was loaded on the SDS-PAGE gel and stained with human TS monoclonal antibody. Pure TS band was observed at 31kDa, no TS band was observed in the control, and a thick band corresponding to the 52kDa size (fusion protein) was observed. We observed another band at 31kDa in this protein which might be associated with a breakdown product from the fusion protein. We will repeat the Western blot and stain with DHFR antibody as well to see any band corresponding to the F/S size can be detected or not.

Specific Aim #3
Transduction of CD34+ cells
Human CD34+ enriched cells were isolated from discarded cancer patients peripheral stem cells mobilized by G-CSF by StemCell Separate kit. The purity of the enriched CD34+ population was above 90% by FACS analysis using anti-human CD34-PE antibody. The separated cells were frozen in liquid nitrogen with 5% DMSO and 90% FBS until used.
CD34+ enriched cells were stimulated with 5 cytokines including recombinant human stem cell factor (rhSCF) 20ng/ml, thrombopoietin (rhTP) 100ng/ml, rhflt-3 100ng/ml, rhGM-CSF10ng/ml, and rhIL-6 100U/ml. After overnight pre-stimulation, cells were infected by supernatants wither SFG-F/S-TS52 (**Table 1**), SFG-EGFP-IRES-F/S-TS52 (**Table 2**), or mock, added 2 times daily for 4 days on Retronectin (Takara Shuzo, Otsu, Japan) coated plates, in the presence of the same concentration of cytokines. Transduced cells were harvested, an aliquot was submitted for FACS analysis and CFU-GM colony assay.

Table 1

	Surviving colonies in 5-FU (M), MTX (2 x 10-8M) or both			
	0	5-FU (10-6M)	MTX (2x10-8)	MTX+5-FU
AM12	100(%)	0	0	0
F/S-TS52	100(%)	36	20	19

The vector transduced and mock transduced human CD34+ enrichced cells were incubated with 5-FU, MTX, or both in the methylcellulose medium with growth factors: rhSCF, rhG-CSF, rhIL-6, rhIL-3 (Moore, MAS, et al). Plates were incubated with 5%CO2 and high humidity. Hematopoietic progenitos were scored at day 14. The values were calculated based on no drug treatment colony numbers as 100%.

Table 2

	Surviving colonies in 5-FU (M)		
	0	10^{-7}	10^{-6}
AM12	100 (%)	8±1	0
EGFP-IRES -F/S-TS52	100 (%)	40±1	19±1

The vector transduced and mock transduced human CD34+ enrichced cells were incubated with 5-FU as described above. The values were calculated based on no drug treatment colony numbers as 100%.The values were mean and SD from 3 experiments.

In Summary, we have accomplished,

1) Retroviral vectors containing a mutated TS and a mutated DHFR or these with EGFP marker both were able to infect NIH3T3 cells and conferred resistance to 5-FU and MTX.
2) The human CD34+ enriched cells infected with these vectors also conferred resistance to 5-FU, MTX, or both 5-FU/MTX combination treatment.

Reportable Outcomes

Abstracts:

N. Takebe, GM Capiaux, SC. Zhao, D. Banerjee, JR Bertino. Generation of fusion protein conferring both methotrexate and fluoropyrimidine resistance in human CD34+ enriched progenitor cells. Blood 94, No10 suppl 1, 1999; 416b.

Gina Marie Capiaux, Naoko Takebe, Debabrata Banerjee, frank Mley, Joseph R Bertino. Generation of a fusion protein conferring both methotrexate and 5-fluoropyrimidine resistance. Proc. of American Association for Cancer Research 41, 2000; 782.

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Abstract# 5086

ΔHGHR, A BIOSAFE CELL-SURFACE LABELLING MOLECULE FOR ANALYSIS AND SELECTION OF GENETICALLY TRANSDUCED HUMAN CELLS. F. Serrano*,[1] M. J. Garcia-Ortiz*,[1] J. L. Abad*,[1] M. A. Gonzalez*,[1] A. Bernad*,[1] (Intr. by Jose L. Diez-Martin) [1]*Immunology and Oncology, Centro Nacional de Biotecnología, Madrid, Spain.*

We describe a new selectable marker for retroviral transduction and selection of human and murine cells. The molecule expressed in the cell surface of the transduced population is a truncated version of the human growth hormone receptor (ΔhGHR), capable of human growth hormone (hGH) binding but devoid of the domains involved in signal triggering. We demonstrate that the engineered molecule is stably expressed in the target cells as an inert molecule unable to trigger proliferation, or rescue from apoptosis upon ligand binding. This new marker will have a tentative wide application spectrum because hGHR in human adult is only highly expressed in liver cells.hGHR is not expressed in hematopoietic tissue, in vitro cultured hematopoietic progenitors or engrafted hu/scid hematopoietic chimeras. The ΔhGHR label has improved biosafety over previously described surface labeling molecules. It belongs to a well characterized hormonal system non-essential in adults and there is extensive clinical experience with hGH administration in humans. This record allows us to anticipate the lack of relevant clinical consequences of massive expression of the transgene as a result of successful replacement of a large tissue with genetically transduced cells. Taking advantage of the differential binding properties of several monoclonal antibodies described, we discuss a cell-rescue method in which the antibody used to select ΔhGHR-transduced cells is eluted by competition with hGH or, alternatively, biotinilated hGH is used to capture tagged cells. To our knowledge this is the first description of a murine antibody-free system to capture retroviral transduced hematopoietic cells.

Abstract# 5087

RECOMBINANT ADENO-ASSOCIATED VIRUS MEDIATED GENE TRANSFER INTO HUMAN PRIMARY LYMPHOCYTES. Noriko Suzuki*, Koichi Miyake*, Yukihiko Hirai*, Takashi Shimada*. (Intr. by Atsushi Manabe) *Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan.*

Peripheral blood lymphocytes are important target cells for gene therapy of various disorders including neoplastic, infectious, and genetic diseases. However, lymphocytes are quite resistant to transduction with currently used viral vectors such as amphotropic retroviral vectors and adenoviral vectors. Recently, we have shown that HIV vectors pseudotyped with HIV envelope are capable of targeted and efficient gene transfer into $CD4^+$ T lymphocytes including non-stimulated primary cells. In this study, we developed a new strategy of preparation of high titer adeno-associated virus (AAV) vectors and explored the ability of the AAV vector to introduce genes into human primary lymphocytes. The AAV vector carrying the CAG promoter driven enhanced green fluorescent protein (EGFP) gene was generated by co-transfection of adenovirus infected 293 cells with the packaging and vector plasmids and was successfully concentrated by the combination of ammonium sulfate precipitation, CsCl, gradient centrifugation, and ultrafiltration using CENTRICON 30 column. The titer of the final preparation was 10^{13} particle/ml and the yield was more than 83 %. Using the high titer AAV vector, we transduced human primary lymphocytes. At a multiplicity of infection (moi) of less than 10^7, the lymphocytes were not transduced with the AAV vector. However, when the lymphocytes were stimulated with PHA and IL2, and incubated with the AAV vector at an moi of $5x10^8$, 22-36 % of the cells were transduced. Transduction efficiencies of $CD4^+$, $CD8^+$, and $CD19^+$ lymphocytes ranged between 24-36%, 19-32%, and 8-25%, respectively. Moreover, 7-12% of freshly isolated non-stimulated primary lymphocytes were transduced with the AAV vector. These results suggest that high titer AAV vectors are useful for lymphocyte gene therapy, and support the development of the further improved packaging system for large scale preparation of high titer AAV vectors.

Abstract# 5088

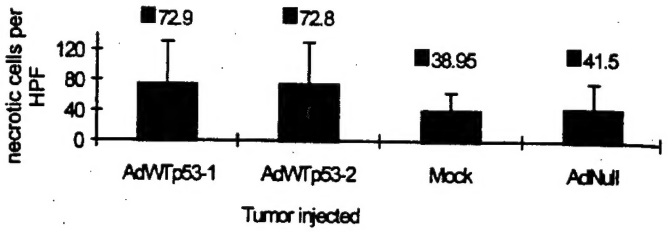
GENERATION OF FUSION PROTEIN CONFERRING BOTH METHOTREXATE AND FLUOROPYRIMIDINE RESISTANCE IN HUMAN CD34+ ENRICHED PROGENITOR CELLS. N. Takebe, G. M. Capiaux*, S. C. Zhao*, D. Banerjee*, J. R. Bertino. *Memorial Sloan-Kettering Cancer Center, New York, NY.*

We previously reported that protection of mouse bone marrow from toxicity caused by methotrexate (MTX) via retroviral transduction (SFG vector) of double mutated human dihydrofolate reductase (DHFR, phenylalanine22/series31;F/S) (Erciklan-Abali, EA et al. Cancer Res 56;4142, 1996) cDNA and generation of a human thymidylate synthase (TS) variant G52S which conferred resistance to fluorodeoxyuridine (FdUrd) (Tong Y, et al. J Biol Chem 273;1161,1998). As MTX and 5-fluorouracil (5-FU) are commonly used chemotherapy drugs, we generated a retroviral vector containing a fusion gene of DHFR(F/S) and TSG52S (TS52). NIH 3T3 cells transduced with SFG-F/S-TS52 conferred resistance to MTX (>200-fold), 5-FU (>10-fold), and FdUrd (>10-fold) compared to control by clonogenic assay. Western blot analysis using human TS antibody confirmed the presence of the fusion protein in NIH3T3 transduced cells. CFU-GM assay using human CD34+ enriched cells transduced by co-culture method with an amphotropic producer cell line (AM12-SFG-F/STS52) showed 36% (5-FU, 10^{-6}M), 20% (MTX, $2 x 10^{-8}$ M), and 19% (5-FU10^{-6} M plus MTX $2 x 10^{-8}$M) surviving colonies in the transduced group compared to no surviving colonies observed in the mock transduced groups. These results encourage the future study of this construct to protect patients from hematologic toxicity of both 5-FU and MTX.

Abstract# 5089

***IN VIVO* BYSTANDER EFFECT OF P53 GENE EXPRESSION MEDIATED BY ADENOVIRAL VECTOR IN AN ANAPLASTIC LARGE CELL LYMPHOMA MODEL IN ATHYMIC MICE.** F. Turturro, T. F. Drevyanko*, C. J. Link*. *Human Gene Therapy Research Institute, John Stoddard Cancer Center, Des Moines Veterans Administration Medical Center, Des Moines, IA, United States.*

Bystander effects using the HSV-tk and ganciclovir system have been well characterized *in vitro* and *in vivo*. This system in part is dependent upon gap junction mediated passage of the toxic metabolite between tumor cells. Although adenoviral p53 mediated cell death is not toxin-related, a bystander effect that amplifies apoptosis has been shown recently *in vitro* with epithelial tumor cells. An antiangiogenic effect of p53 protein has also been documented and proposed as an additional mechanism of bystander effect. We have recently established human anaplastic large cell lymphoma derived xenografts in athymic nude mice. There is significant difference in the number of necrotic cells per high power field in six tumors injected with AdWTp53 (adenovirus expressing p53) when compared to those injected with AdNull (adenoviral backbone) or mock infected.



Analysis of slides from the same tumors by TUNEL assay demonstrated that apoptosis was only present in tumors injected with AdWTp53 and was confined to tumor areas around the needle track. Interestingly, only in AdWTp53 injected tumors, additional areas of necrosis were observed more distant from the needle track that were negative by TUNNEL assay. Necrosis was defined as either pyknosis, karyorrhexis, karyolysis or coagulation necrosis. This observation suggests *in vivo* bystander effect that warrants further investigation to elucidate the mechanism of the indirect cell killing, that results in necrosis in addition to the previously described local apoptosis.

Abstract# 5090

***IN VIVO* DIFFERENTIATION OF MESENCHYMAL STEM CELLS INTO ADIPOCYTES FOR TISSUE-SPECIFIC ANTIGEN PRESENTATION.** P. Vanguri*,[1] L. Tao*,[1] S. Beck*,[1] R. K. Jaiswal*,[1] M. F. Pittenger*,[1] D. R. Marshak*,[1] K. R. McIntosh.[1] [1]*Osiris Therapeutics, Inc., Baltimore, MD.*

Human mesenchymal stem cells (hMSCs) genetically engineered to express B7 costimulatory molecules and treated with IFN-γ to up-regulate MHC class II can function as antigen presenting cells. Since hMSCs can differentiate into various cell types, the modified cells could be developed for tissue-specific antigen presentation. We previously demonstrated antigen presentation function of hMSC-derived adipocytes *in vitro*. In this study, differentiation of MSCs into adipocytes was characterized *in vitro* and *in vivo* with the goal of regulating gene expression in a tissue-specific manner. Treatment of hMSCs with an adipogenic medium containing dexamethasone, isobutyl methyl xanthine, insulin and indomethacin, resulted in a population of adipocytes consisting of cells with negligible to large accumulations of fat droplets. Committed adipocytes expressed mRNAs for adipose-specific genes, including fatty acid binding protein (aP2), lipoprotein lipase, peroxisome proliferator-activated receptor-γ and leptin detected by RT-PCR, whereas they were not found in undifferentiated cells. The aP2 promoter was selected as suitable for adipose-specific expression of proteins. We constructed an 800 bp promoter by joining 300 bp of the proximal promoter to 500 bp of adipose-specific enhancer regions from the aP2 gene. Using an aP2-promoter-Green Fluorescent Protein (GFP) reporter construct we showed significant expression of GFP by transfected hMSC-derived adipocytes while expression by untreated hMSCs was negligible. Differentiation of MSCs into adipocytes *in vivo* was studied in nude rats and mice. hMSCs were labeled with the persistent fluorescent dye CM-DiI, then transplanted into the sternal fat pad of nude rats. The transplanted cells differentiated into adipocytes in 4 to 6 weeks. In a mouse model, murine cells were loaded into gelatin implants and placed into subcutaneous fat tissue. Implanted cells differentiated into adipocytes within a week. By 6 weeks, numerous adipocytes were found attached to the gelatin fibrils surrounded by a fibrocytic capsule, indicative of long-term survival of the cells. We have demonstrated adipose-specific gene expression using the aP2 promoter and adipose tissue-specific differentiation of hMSCs *in vivo*. Since hMSC-derived adipocytes were previously also shown to function as antigen presenting cells, the opportunity now exists to utilize hMSCs for tissue-specific antigen presentation. Since adipose tissue is abundant and highly vascularized, adipocytes differentiated from hMSCs *in situ* could potentially serve as efficient vehicles for delivery of vaccines or therapeutic proteins.

#4971 GENERATION OF A FUSION PROTEIN CONFERRING BOTH METHOTREXATE AND 5-FLUOROPYRIMIDINE RESISTANCE. Gina Marie Capiaux, Naoko Takebe, Debabrata Banerjee, Frank Maley, and Joseph R Bertino, *Memorial Sloan-Kettering Cancer Ctr, New York, NY, and New York State Dept of Health, Wadsworth Ctr, Albany, NY*

Dihydrofolate reductase (DHFR) and thymidylate synthase (TS), two key enzymes responsible for purine and thymidylate synthesis respectively, are common targets for chemotherapeutic agents such as methotrexate (MTX) and 5-fluorouracil (5-FU). However, despite their efficacy in the treatment of a variety of cancers, the use of MTX and 5-FU are limited by their myelosuppressive and gastrointestinal toxicity. We have previously generated drug-resistant mutants of each of these enzymes for use in gene therapy based myeloprotection strategies, and have demonstrated drug resistance in mouse and human bone marrow. As MTX and 5-FU are used in combination therapy and a single bifunctional enzyme containing both DHFR and TS exists naturally in protozoans such as Leischmania and Cryptosporidium, we have generated a fusion protein of the two mutant enzymes. Here, we have demonstrated that a fusion protein of DHFR (L22F-F31S) and TS (G52S) is functional with kinetic characteristics similar to that of the individual mutant enzymes as well as demonstrate its resistance to MTX and 5-FU in human CD34+ enriched progenitor cells.